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The Maize *Dwarf3* Gene Encodes a Cytochrome P450-Mediated Early Step in Gibberellin Biosynthesis

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Gibberellins (GAs) are phytohormones required for normal growth and development in higher plants. The *Dwarf3* (*D3*) gene of maize encodes an early step in the GA biosynthesis pathway. We transposon-tagged the *D3* gene using Robertson's *Mutator* (*Mu*) and showed that the mutant allele *d3-2::Mu8* is linked to a *Mu8* element. The DNA flanking the *Mu8* element was cloned and shown to be linked to the *d3* locus by mapping in a high-resolution population developed by selecting for recombination between *d3* and linked genetic markers. To establish unambiguously the identity of the cloned gene as *D3*, a second mutant allele of *D3* (*d3-4*) was also cloned and characterized using the *d3-2::Mu8* sequences as a probe. *d3-4* was found to have a novel insertion element, named *Sleepy*, inserted into an exon. A third mutant allele, *d3-1*, which has the same size 3′ restriction fragments as *d3-4* but different 5′ restriction fragments, was found to contain a *Sleepy* insertion at the same position as *d3-4*. On the basis of the pedigree, *Sleepy* insertion, and restriction map, *d3-1* appears to represent a recombinational derivative of *d3-4*. The *D3* gene encodes a predicted protein with significant sequence similarity to cytochrome P450 enzymes. Analysis of *D3* mRNA showed that the *D3* transcript is expressed in roots, developing leaves, the vegetative meristem, and suspension culture cells. We detected reduced *D3* mRNA levels in the mutant allele *d3-5*.

INTRODUCTION

Gibberellins (GAs) are isoprenoid phytohormones required for shoot elongation in higher plants, and it has been proposed that they act as signals in other processes, such as germination, juvenile-to-adult transitions, vernalization, and flowering (Jones, 1973; Koornneef and van der Veen, 1980; Pharis and King, 1985; Reid, 1986). The essential role for GAs in shoot elongation has been demonstrated clearly by the isolation of mutants deficient in GA biosynthesis in a number of plant species, including maize, pea, tomato, Arabidopsis, and rice. Mutants in all of these species typically have shortened internodes, resulting in a dwarf phenotype (Reid, 1986). The roles of GAs in other developmental processes, such as vernalization and flowering, are less well understood.

Maize is an attractive system for the analysis of GA biosynthesis because five nonallelic dwarf mutants that are blocked in biosynthetic steps have been isolated (Phinney and Spray, 1982; Coe et al., 1988; Fujioka et al., 1988; Bensen et al., 1995), and transposons can be used to identify genes for which tagged mutants have been isolated. For example, the *Anther ear1* gene has recently been cloned by transposon tagging (Bensen et al., 1995). Bioassay experiments (Phinney and Spray, 1982), together with biochemical studies (Hedden et al., 1982; Fujioka et al., 1988; Suzuki et al., 1992), have led to the following proposed pathway of GA biosynthesis for maize: *ent*-kaurene → *ent*-kaurenol → *ent*-kaurenal → *ent*-kaurenoic acid → *ent*-7α-hydroxykaurenoic acid → GA_{12}-aldehyde → GA_{12} → GA_{53} → GA_{44} → GA_{19} → GA_{20} → GA_1. GA_1 has been proposed to be the biologically active GA in maize (Phinney and Spray, 1982).

Here, we report the transposon tagging of the maize *Dwarf3* (*D3*) gene, which encodes an early step in the biosynthesis of GA, probably the 13-hydroxylation step (Phinney and Spray, 1982). The *d3* mutant of maize was first described by Demerec (1926) and shown to be linked to the chromosome 9 marker *shrunken1*. The *d3* locus was later mapped to the interval between *waxy* (*wx*) and *glossy15* (*gl15*) (Coe et al., 1988). *D3* is of particular interest because little progress has been made in isolating any of the genes that encode the cytochrome P450 GA biosynthetic enzymes.

RESULTS

Identification of *d3-2::Mu8*

The Robertson's *Mutator* (*Mu*) family of maize transposons causes a high forward mutation rate resulting in visible mutations in a high percentage of self-pollinated progeny (Chandler and Hardeman, 1991). In a mutagenesis experiment designed to find new mutant phenotypes, a self-pollinated ear from a

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Robertson's *Mu* line segregated one-quarter of the dwarf plants (*d**). This line contained the *Mu*-induced mutable aleurone marker *bronze1–mum9* (*bz1–mum9*) that is commonly used in *Mu* tagging; *bz1* maps to chromosome 9s. A heterozygous plant was crossed to the wild-type inbred T232, and the F_1 generation self-pollinated. In the resulting F_2 progeny, nine of 10 *bz1* kernels gave rise to *d** plants, indicating that *d** maps to chromosome 9. Although other anthocyanin markers were segregating in this cross, *bz* and *Bz* kernels were clearly distinguishable. DNA gel blot analysis of the *T232/d** F_2 progeny (n = 30) using the restriction fragment length polymorphism (RFLP) probes *bnl3.06*, *wx*, *umc81*, and *umc114* showed that *d** maps to chromosome 9c between *bz1* and *umc81* with no recombination observed between *d** and *wx* (data not shown).

The *d** plants had the characteristic features (Phinney, 1956; Coe et al., 1988) of mutants deficient in GA biosynthesis (data not shown): (1) *d** confers a dwarf phenotype, 10 to 20% the height of wild-type plants, and short dark green leaves; (2) plants of *d** can be converted to near normal height by the addition of GA; (3) *d** confers an andromonoecious phenotype. In addition, under Tucson, Arizona, field conditions, *d** plants have >6 tillers and are both male and female sterile. This is also true of *d3* mutant plants when grown under Tucson field conditions (Winkler and Freeling, 1994), although both *d** and *d3* can be crossed if grown in the greenhouse and treated with GA.

Maize *d3*, a GA-responsive dwarf mutant, also maps to chromosome 9c near *wx* (Phinney and Spray, 1982; Coe et al., 1988). To test the hypothesis that *d** is an allele of *d3*, a heterozygous *d** plant (identified by RFLP analysis) was crossed to a homozygous *d3-4* plant treated with GA in the greenhouse. The testcross progeny segregated dwarf and wild-type plants in a 1:1 ratio, indicating allelism with *d3*. This *d3* allele is subsequently referred to as *d3-2::Mu8* because we show that the molecular basis of the mutation is a *Mu8* element inserted into the last exon.

Identification of a Linked *Mu* Transposon

To find candidate-linked transposons, we used bulked segregant analysis (Michelmore et al., 1991). This method allows multiple probe and enzyme combinations to be analyzed for linkage with a minimal amount of material and effort. Leaves of eight homozygous dwarf plants and eight homozygous wild-type plants (identified by RFLP analysis) from the F_2 progeny were used to establish dwarf and wild-type pools of DNA. Bulked segregant analysis using genomic DNA gel blots prepared with four restriction enzymes (BamHI, BclI, BglII, and EcoRI) and probed with internal fragments from all known *Mu* transposons identified four candidate-linked *Mu*-containing fragments; these candidates were analyzed in testcross progeny. Figure 1A shows a 14-kb EcoRI *Mu8*-hybridizing band that was observed present in 30 individual dwarf plants and absent in 28 wild-type plants from testcross progeny (10 individuals shown). This indicates linkage between the 14-kb

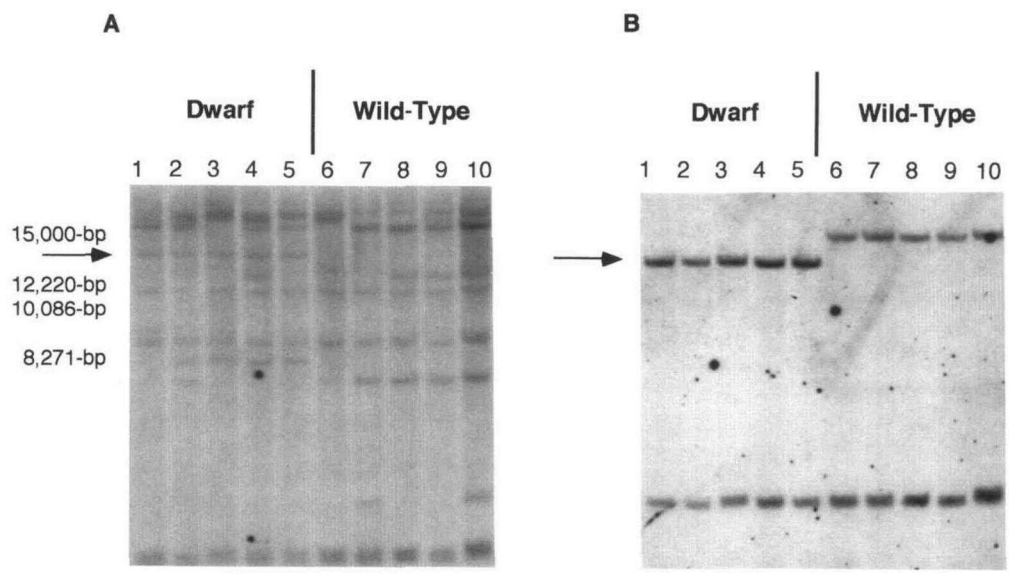

Figure 1. Transposon Tagging of Maize *D3*.

(A) DNA gel blot analysis of the testcross progeny of a *d3-2::Mu8* heterozygous plant × a *d3-4* homozygous plant using the transposon *Mu8* as a probe. DNA from either dwarf plants (lanes 1 to 5) or wild-type plants (lanes 6 to 10) was digested with EcoRI, resolved by agarose gel electrophoresis, blotted onto a nylon membrane, and probed with a digoxigenin-dUTP–labeled *Mu8* probe. The arrow indicates the 14-kb EcoRI fragment, and molecular length markers are shown at left.

(B) DNA gel blot analysis of the testcross progeny of *d3-2::Mu8* heterozygous plants × *d3-4* homozygous plants using an isolated fragment. The blot from **(A)** was stripped and reprobed with subclone 5. The arrow indicates the 14-kb EcoRI fragment.

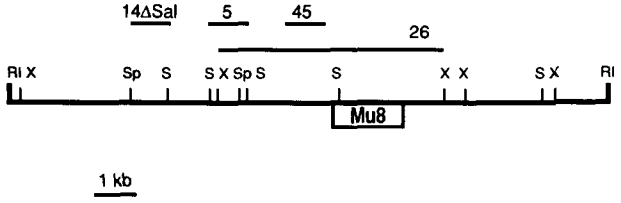

Figure 2. Restriction Map of the 14-kb EcoRI Genomic Clone of *d3-2::Mu8.*

Subclones 14ΔSal, 5, 45, and 26 that were used as probes in hybridization experiments are shown. RI, EcoR; S, SalI; Sp, SpeI; X, XbaI.

EcoRI-*Mu8*–containing fragment and *d3-2::Mu8.* All other candidate *Mu*–linked fragments were shown to recombine in the same testcross progeny.

Cloning of DNA Flanking the *Mu8* Element

To clone the candidate tagged *d3* gene, a size-fractionated EcoRI subgenomic library was prepared from homozygous *d3-2::Mu8* plants in λEMBL4 using ER1647 (*recD*$^-$) as a host, and plaque lifts were probed with *Mu8.* Figure 2 shows the restriction map of the positive 14-kb clone λ-Zmd3.2. When the genomic DNA gel blots from the testcross were hybridized with probe 5 (Figure 2), it was observed that the fragments comigrated with the originally observed *Mu8* band (Figure 1B). In addition, no recombination was found between the *d3* locus and the cloned fragment when DNA from 16 homozygous *d3-2::Mu8* plants and an additional 64 plants from the testcross progeny were examined by DNA gel blotting (data not shown). A previous screening of a library (500,000 primary plaques) prepared from the same ligation reaction in the host XL1-Blue was unsuccessful, suggesting that *Mu8*-containing clones may be unstable in some cloning vector–host combinations. Both strains are defective for several enzymes that cleave methylated sequences. *recD*$^-$ hosts stabilize some sequences that are unstable in other hosts (Wyman and Wertman, 1987).

Linkage to *D3*

To test critically the linkage of the cloned fragment to the *d3* locus, the clone was mapped in a set of recombinant inbred progeny and a high-resolution mapping population. These results are summarized in Figure 3. Subclone 5 maps to chromosome 9 in the interval between *wx* and *umc81* in the CM × T recombinant inbred lines (n = 48) developed by Burr et al. (1988) (Figure 3A). Because the kernel marker *wx* and the seedling marker *gl15* are closely linked to *d3*, a heterozygous triple mutant stock *wx d3-4 gl15/Wx+ D3+ Gl15+* was self-pollinated, and recombinants between the two intervals, *d3* to *wx* and *d3* to *gl15*, were selected. Examination of progeny to test 600 chromosomes for recombination revealed 15 recombinations each between *d3* to *wx* and between *d3* to *gl15.* DNA was prepared from these 30 recombinant individuals and subjected to DNA gel blot analysis using probes 5, *wx*, and *umc114.* No recombination was observed between *d3* and the cloned fragment (Figure 3B). In these and the previously described testcross and F_2 progeny, a total of 754 chromosomes were examined, and no recombination between the cloned fragment and *d3* was observed, indicating tight linkage of the clone to the *d3* locus.

Maize D3 Is a Cytochrome P450

To identify the nature of the *D3* gene product, cDNAs were isolated from a light-grown seedling library and a vegetative meristem library by using our genomic subclone 26 as a probe. A resulting 1.4-kb clone was sequenced. Polymerase chain reaction (PCR) analysis using primers designed from the 5′

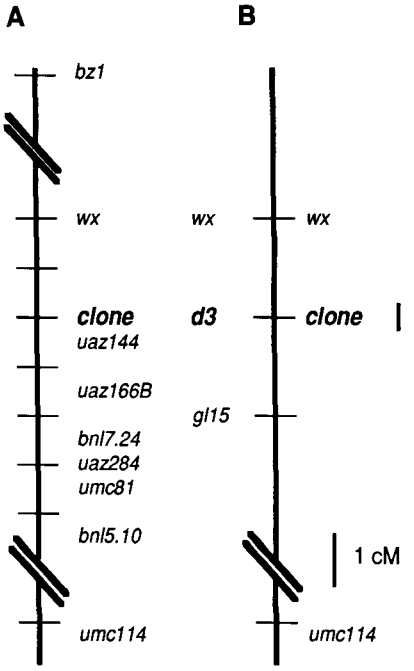

Figure 3. Genetic Mapping of the *D3* Candidate Clone.

(A) The candidate *D3* clone was mapped to chromosome 9 near *wx* in the CM × T recombinant inbred population. Some linked RFLP loci are shown on the right for reference.

(B) F_2 self-progeny of the triple mutant *wx d3-4 gl15/Wx+ D3+ Gl15+* were planted, and recombinant classes were selected. DNA was prepared from recombinant individuals and analyzed on DNA gel blots. RFLP loci analyzed in this population are shown on the right, and genetic markers analyzed in this population are shown on the left. No recombination was observed between the *d3* locus and the candidate clone in a population of 600 chromosomes that were analyzed for recombination. cM, centimorgan.

sequence and the vector was used to identify longer *D3* clones that were positive in the primary screen but not purified initially. A longer clone was purified, and its 5′ end was sequenced. The combined sequence of the two longest cDNA clones is 1692 bp but appears to be short of full length. Sequence analysis of the *d3-2*::*Mu8* genomic clone revealed that there are two in-frame ATGs 16 and 31 bp upstream of the 5′ end of the longest cDNA; the second ATG is in better context (Joshi, 1987). A consensus TATA box (TATATA; Joshi, 1987) is located 150 bp upstream of the first in-frame ATG (data not shown). Although these results are consistent with the cDNA being nearly full length, this hypothesis requires experimental verification. Figure 4 shows the combined sequence of the cDNA clones and 31 bp of the genomic sequence 5′ of the longest cDNA. If the first ATG from the *d3-2*::*Mu8* genomic sequence is the initiation codon, a single open reading frame of 1560 bp would be predicted (encompassing nucleotides 1 to 1560). The predicted 519–amino acid D3 protein would have a molecular mass of 57.9 kD. Three potential polyadenylation sites (Hunt, 1994) were identified, although all four identified cDNA clones had identical 3′ ends.

Data base comparisons showed that the predicted D3 protein has significant sequence similarity to the cytochrome P450 gene superfamily, as shown in Figure 5. It has the highly conserved cytochrome P450 signature sequence (FxxGxxxCxG) (where x is a non-consensus amino acid). The cysteine of the signature sequence is involved in binding heme Fe (Nebert and Gonzalez, 1987; Porter and Coon, 1991). As has been observed generally, the N terminus has fewer sequence identities to known P450 proteins (Nebert and Gonzalez, 1987). In contrast, the C-terminal 175 amino acids showed ~20% identity to at least 20 known cytochrome P450 sequences; many of these are involved in mammalian steroid metabolism. The sequence similarity is primarily in the four segments of sequence similarity described by Kalb and Loper (1988). In addition, the amino acid sequences MxYLxVxxETLR in the B domain and GYxxIPKG in the C domain are shared with a number of related cytochrome P450s. The closest related cytochrome P450 that has been fully sequenced has <40% amino acid identity to D3; therefore, D3 defines a new cytochrome P450 family in accordance with the guidelines suggested for cytochrome P450 nomenclature (Nebert and Nelson, 1991). D3 has been named CYP88 (D. Nelson, cytochrome P450 nomenclature committee). However, the translation of a partial Arabidopsis cDNA sequence recently submitted to GenBank as an expressed sequence tag (T43711) shows 62% amino acid identity to D3.

Several other features of the predicted D3 protein deserve mention. Hydrophobicity plotting of D3 showed that the N-terminal amino acid sequence of D3 is hydrophobic, as expected, given its presumed microsomal localization (data not shown). In addition, amino acids 9 to 35 and 37 to 63 are an imperfect repeat with five amino acid differences; a repeat such as this has not been observed in other cytochrome P450 sequences. In addition, the sequence AARRA is repeated three times in the N-terminal region of the protein.

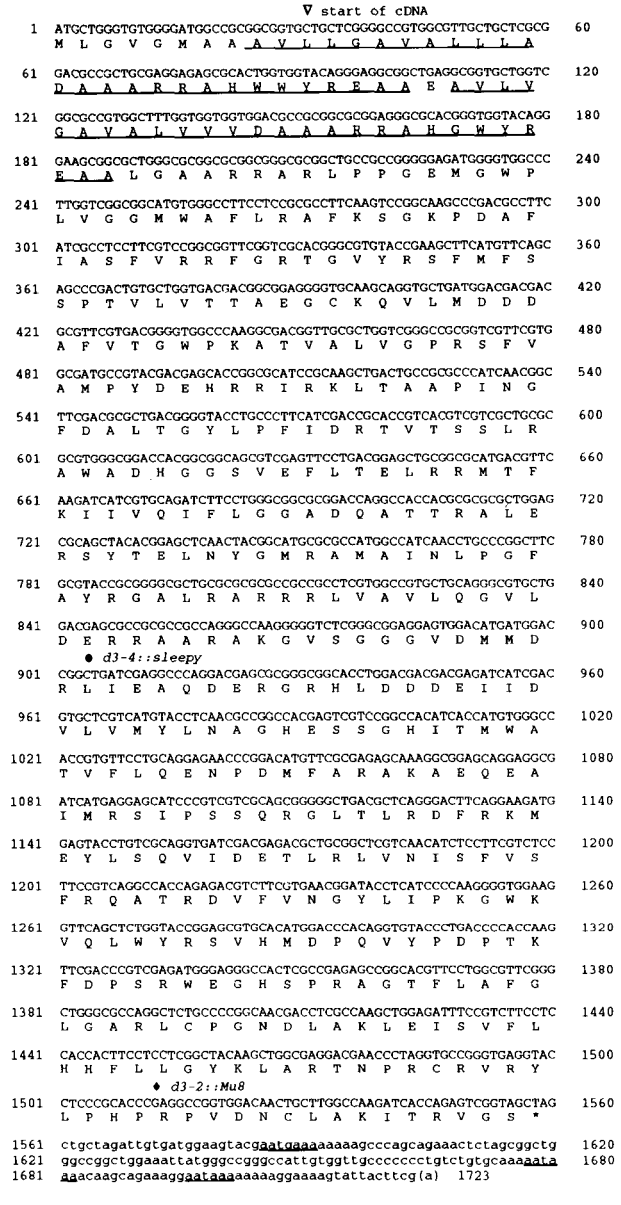

Figure 4. Sequence of the *D3* Gene.

The nucleotide sequence of the *D3* gene and the predicted amino acid sequence of the corresponding protein are shown. The open reading frame of the *D3* gene is shown in uppercase letters; the 3′ untranslated sequence is shown in lowercase letters. The first 31 bp are derived from the genomic sequence of the *d3-2*::*Mu8* allele. The start of the cDNA, which is probably short of full length, is indicated by an arrowhead above the sequence. The sites of the *d3-2*::*Mu8* and *d3-4*::*Sleepy* insertions are shown with diamonds above the sequence. The imperfect repeats at the N terminus are underlined. The termination codon is indicated by an asterisk. Three potential polyadenylation sites are underlined.

Identification of the *d3-2*::*Mu8* Mutation Site and a Novel Insertion in the Mutant Allele *d3-4*

The DNA flanking the *d3-2*::*Mu8* insertion was sequenced to determine the site of the *d3-2*::*Mu8* mutation. Figure 6A shows the position of the *Mu8* insertion, which is located in the last exon and disrupts the predicted protein 16 amino acids from the C terminus. Nine–base pair direct repeats flanking the *Mu8* insertion were found and are characteristic of *Mu* insertion sites (Chandler and Hardeman, 1991). The first G residue of the direct repeat of the *d3-2*::*Mu8* allele is polymorphic, in contrast with the sequence of the *B73* allele (cDNA); however, the predicted translation of this polymorphism is silent.

DNA gel blot analysis indicated that six *d3* stocks in our collection were most likely different alleles. DNA from dwarf plants of each *d3* allele and 15 wild-type lines was digested with XbaI, HindIII, and EcoRI and analyzed on genomic DNA gel blots using 5′ genomic probe 5 (first intron) or 14ΔSal (putative promoter region) and the 3′ cDNA probe 45. On the basis of the RFLPs, six different *d3* alleles were identified. Table 1 gives a partial summary of these results. Analysis of the Maize Genetics Cooperation Stock Center pedigrees showed that at

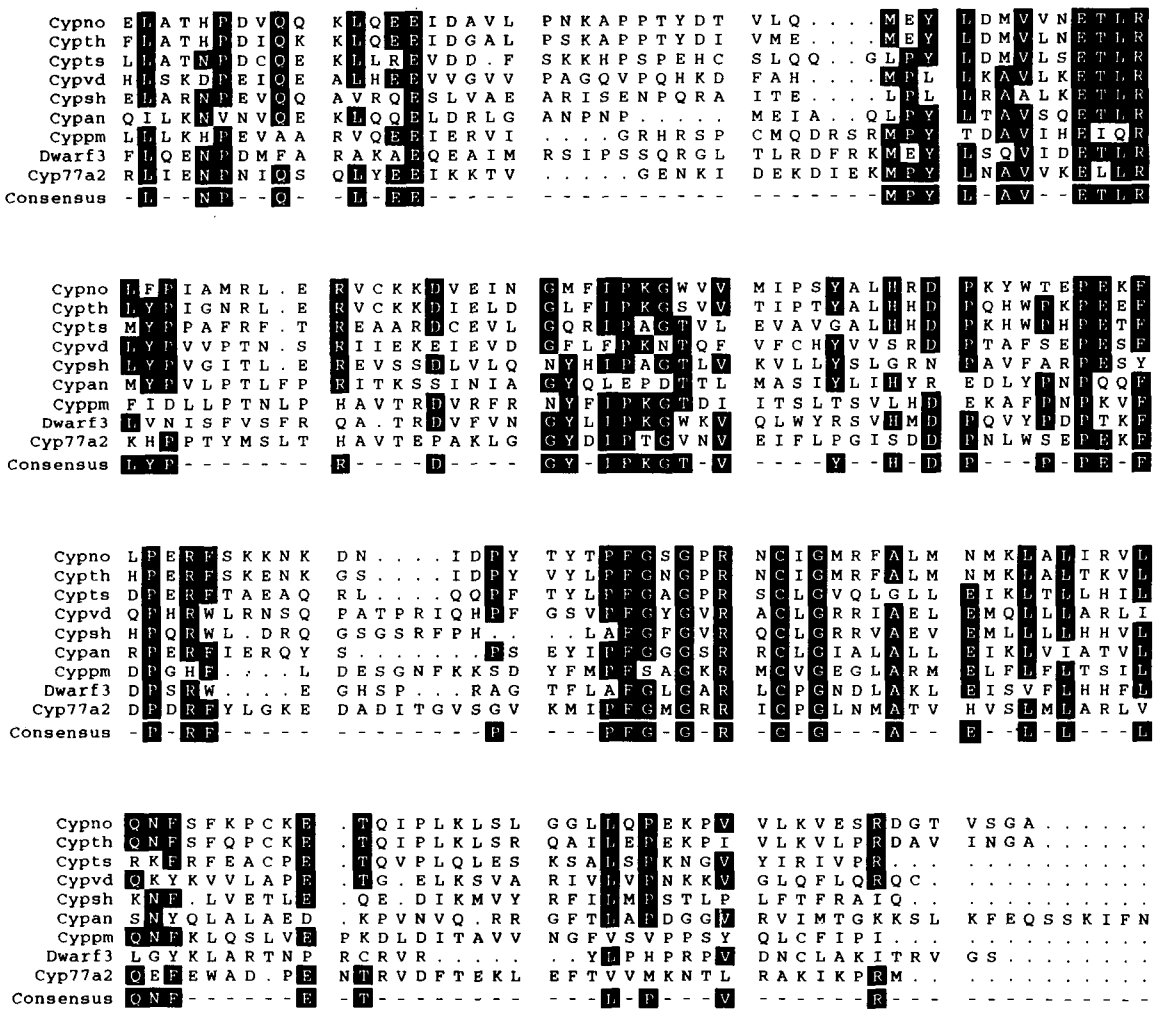

Figure 5. Multiple Alignment of the C-Terminal 175 Amino Acids of D3 and Cytochrome P450s with Sequence Similarity.

The amino acids identical to the consensus sequence are shaded in black; the bottom line indicates the consensus sequence. Gaps introduced to improve the alignment are denoted by dots; dashes were used where there was no consensus sequence. Cypno, human nifedipine oxidase; Cypth, rat testosterone 6β-hydroxylase; Cypts, pig thromboxane synthase; Cypvd, human vitamin D3-25-hydroxylase; Cypsh, bovine steroid 11β-hydroxylase; Cypan, probable P450 of *Anabaena*; Cyppm, rabbit progesterone monooxygenase; Dwarf3, maize D3; Cyp77a2, eggplant cytochrome P450.

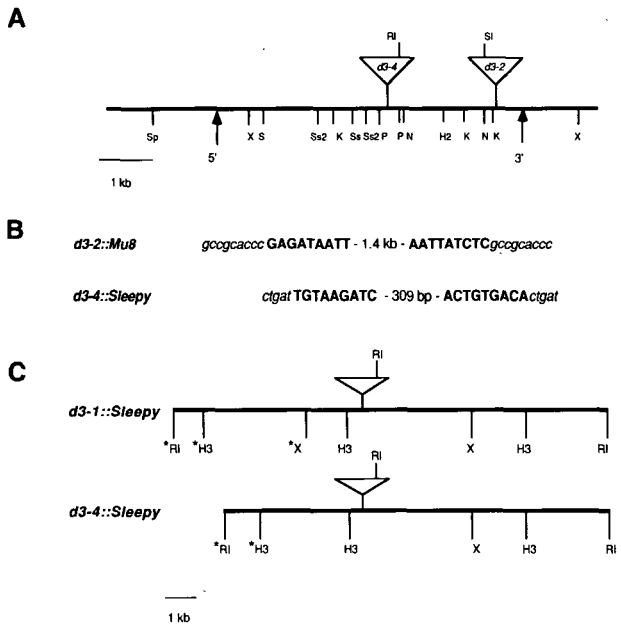

Figure 6. Identification of Mutations in *d3-2::Mu8* and *d3-4* and *d3-1*.

(A) Restriction map of the *d3* locus. The sites of insertions for the mutant alleles *d3-2::Mu8* and *d3-4* are shown as triangles. The 5′ end of the longest cDNA is indicated with an arrow, and the 3′ end of the mRNA is indicated with an arrow labeled 3′. H2, HindII; K, KpnI; N, NruI; P, PstI; S, SalI; Sp, SpeI; Ss, SstI; Ss2, SstII; X, XbaI.

(B) The direct repeats surrounding the insertion sequences are shown in lowercase letters. The terminal nine base pairs of the insertion elements are shown in uppercase letters.

(C) The restriction maps of *d3-1* and *d3-4* are shown; polymorphic sites are marked with asterisks. H3, HindIII; RI, EcoRI; X, XbaI.

least five *d3* alleles have been donated (P. Stinard, personal communication). The allele in the marker stock *wx d3 gl15* was named *d3-4*. *d3-4* was also identified in an independently maintained stock that had been backcrossed into inbred A188 seven times.

To confirm further the identity of the isolated gene as *D3*, we identified the mutation in the *d3-4* mutant allele. Genomic DNA gel blot analysis showed that *d3-4* individuals have a unique 5-kb EcoRI fragment. This band was not observed in any of a total of 23 wild-type lines analyzed by probing genomic DNA gel blots with probe 5. The 3′ cDNA probe 45 identified a 9-kb band for the *d3-4* allele on the same blots (Table 1). This suggests that there is a novel EcoRI site in the *d3-4* allele. One explanation for the novel EcoRI site would be that an insertion element containing an EcoRI site had been inserted into the *d3-4* allele. The 5-kb EcoRI fragment of *d3-4* was cloned into λZAPII. A 458-bp SstI-to-EcoRI subclone containing the novel EcoRI site in the *d3-4* allele was sequenced. The sequences of the cDNA clone, *d3-2::Mu8*, and *d3-4* were identical for 175 bp, beginning from the SstI site. The 283 bp on the 3′ end of the *d3-4* genomic clone showed no sequence similarity to either the sequenced region of the *d3-2* genomic clone or the cDNA clone. This is consistent with an insertion.

An "insertion"-specific primer (G5) and a 3′ *D3* primer (G6) were designed to amplify the 3′ end of the putative insertion in *d3-4*; the 3′ end of the sense G5 primer was the first G residue of the novel EcoRI site, and G6 was an antisense primer identical to base pairs 1180 to 1200 of the predicted coding region (Figure 4). PCR amplification yielded an ~550-bp band that was cloned and sequenced (three independent clones). Sequence analysis of this clone showed that 44 bp 3′ of the EcoRI site are not related to the *d3-2::Mu8* genomic sequence in this region or to the cDNA sequence. Flanking the novel 328 bp of sequence are 5-bp direct repeats (Figure 6B). Duplication of sequences surrounding an insertion site is a characteristic feature of many transposable element insertions. In addition, the ends of the insertion consist of inverted repeats, which are also a characteristic feature of transposable elements, although in this case 3-bp inverted repeats are exceptionally short. When the insertion was used as a probe to analyze a genomic DNA gel blot of four maize inbred lines, it hybridized to three to four fragments that did not comigrate with the *D3* fragments identified by probe 5 or probe 45 (data not shown). The 328-bp insertion of *d3-4*, here named *Sleepy*

Table 1. Summary of the RFLPs of Six Mutant Alleles of the *d3* Locus

		EcoRI		HindIII		XbaI	
Allele	Mutation	5′ Probe[a] (kb)	3′ Probe[b] (kb)	5′ Probe[a] (kb)	3′ Probe[b] (kb)	5′ Probe[c] (kb)	3′ Probe[b] (kb)
d3-1	*Sleepy*	6.5	9.0	5.2	8.0	2.8	9.0
d3-2::Mu8	*Mu8*	14	14	4.7	—	5.8	5.5
d3-4	*Sleepy*	5.0	9.0	3.8	8.0	12	12
d3-5	—[d]	15	—	5.8	—	3.6	5.5
d3-6	—	10.5	—	7.0	—	2.7	—
d3-660B[e]	—	12	12	—	—	4.5	—

[a] Probe 5.
[b] Probe 45.
[c] Probe 14ΔS.
[d] Dashes indicate not determined.
[e] Ethyl methanesulfonate was used as a mutagen in the isolation of *d3-660B*.

(GenBank accession number U28041), resides in an exon and has no significant similarity to any other sequences in the data base.

An allele-specific PCR assay using the *d3*-4 allele–specific PCR primer pair G5/G6 was employed to examine all *d3* stocks. A *d3* allele backcrossed into the inbred A188 background seven times also had the characteristic *d3-4* 550-bp PCR band and an identical restriction map, indicating that it is identical to *d3-4*. *d3-1* also had a 550-bp band when PCR amplified with the G5/G6 primer pair (Figure 6C). Comparison of *d3-4* and *d3-1* by genomic DNA gel blots showed that the EcoRI, HindIII, and XbaI restriction fragments on the 3′ end of the gene were identical, but that the EcoRI, HindIII, and XbaI restriction sites 5′ of the *Sleepy* insertion were polymorphic (Table 1). These results suggest that the *d3-1* and *d3-4* alleles have a *Sleepy* insertion at the same position but have distinct 5′ ends. Analysis of the pedigree of *d3-1* and *d3-4* indicated that they can be traced back to the same progenitor *d3* stock grown in 1953 at the Maize Stock Center (P. Stinard, personal communication). The simplest explanation for these results is that the *d3-1* allele is a recombinational derivative of *d3-4*. Recombinational derivatives would be expected to be very rare if recombination was uniform throughout the maize genome. However, it has been proposed that because the length of total chromosome maps is fairly constant among eukaryotes, recombination may be confined to structural genes (Thuriaux, 1977). In addition, analysis of recombination at the maize *a1* and *bz1* loci has indicated that at least in these two cases in maize, structural genes may be recombinational hot spots (Dooner, 1986; Brown and Sundaresan, 1991).

Analysis of *D3* mRNA Expression

Maize *D3* mRNA expression was observed in roots and pooled leaves of 7-day-old plants by using reverse transcription–PCR (RT-PCR) as shown in Figure 7. In 3-week-old plants, *d3* mRNA expression was observed in young developing leaves (~2 cm in length) and the vegetative meristem as well as in NO_3-induced suspension culture cells (Figure 7). *D3* mRNA expression was not observed in the mutant alleles *d3-5* in developing leaves with the G1/G2 primer pair (data not shown). RT-PCR was not a quantitative analysis as performed.

DISCUSSION

We cloned the *D3* gene of maize that encodes one of the early steps in GA biosynthesis. The identification of the *D3* gene was established, in part, by the molecular characterization of three mutant alleles. The allele *d3-2*::*Mu8* has a *Mu8* insertion in an exon that disrupts the 3′ end of the *D3* transcript. A second mutant allele, *d3-4*, possesses a novel insertion, named *Sleepy*, in an exon. A third mutant allele, *d3-1*, which has the same size 3′ restriction fragments as *d3-4* but different 5′

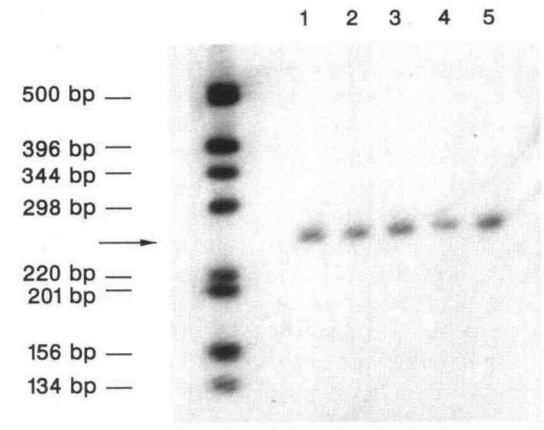

Figure 7. RT-PCR Analysis of Maize *D3* Expression.

First-strand cDNAs from roots (lane 1), young developing leaves ~2 cm in length (lane 2), the vegetative meristem (lane 3), young developing leaves of 3-week-old plants (lane 4), and NO_3-induced suspension culture cells (lane 5) were amplified by PCR with the *D3*-specific primer pair G3/G4, resolved by agarose gel electrophoresis, blotted onto a nylon membrane, and probed with digoxigenin-dUTP–labeled *D3* probe 45. The arrow indicates the 258-bp fragment detected by the *D3* probe. Molecular length markers are shown at left.

restriction fragments, was also found to contain a *Sleepy* insertion element by using PCR. On the basis of the pedigree and restriction map, *d3-1* appears to represent a recombinational derivative of *d3-4*. In addition, the molecular characterization of a fourth mutant allele, *d3-5*, showed that *d3-5* has levels of mRNA expression in leaves below the detection limit of RT-PCR. The identification of the *D3* gene was also supported by the observed lack of recombination between the cloned fragment and the *d3* locus. No recombination between the *d3* locus and the cloned fragment was observed in 754 chromosomes. Although the observed lack of recombination does not provide direct proof for the hypothesis that the cloned fragment is *D3*, it is a very strong negative test of this hypothesis. The predicted D3 protein has significant sequence similarity to members of the cytochrome P450 gene superfamily, as predicted by its proposed position in the GA biosynthesis pathway.

Biochemical analysis and bioassay data indicate that the *d3* mutation blocks an early step in the pathway of GA biosynthesis. In bioassay experiments, maize *d3* plants yield a positive growth response to GA_1, GA_{20}, GA_{53}-aldehyde, and GA_{53} but not to *ent*-kaurene or GA_{12}-aldehyde (Phinney and Spray, 1982). However, caution must be used in interpreting the lack of growth response of *d3* plants to GA_{12}-aldehyde because the growth response of *d5* plants to GA_{12}-aldehyde is ~5% of the growth observed for *d3* or *d5* plants with GA_{20} (maize *d5* is defective in *ent*-kaurene synthesis). Biochemical analyses indicate that levels of the GA biosynthetic intermediates GA_{53}, GA_{19}, and GA_{20} as well as bioactive GA_1 are reduced in *d3* plants (Fujioka et al., 1988). Together these results are consistent with the *D3* gene encoding an early 13-hydroxylase activity

($GA_{12} \rightarrow GA_{53}$) and are critical for the proposal that in maize, an early 13-hydroxylation is the major pathway used for the biosynthesis of GA_1, which is required for shoot elongation.

The enzymatic catalysis of the five consecutive oxidation steps early in the biosynthesis of gibberellins—*ent*-kaurene → *ent*-kaurenol → *ent*-kaurenal → *ent*-kaurenoic acid → *ent*-7α-hydroxykaurenoic acid → GA_{12}-aldehyde—is microsomal and requires NADPH characteristic of cytochrome P450 enzymes (West, 1980; Hedden, 1983; Graebe, 1987). The oxidative pathway from *ent*-kaurene to GA_{12}-aldehyde is identical in all plant species examined to date and is thought to be universal (Hedden, 1983; Graebe, 1987). The GA biosynthesis pathway after the biosynthesis of GA_{12}-aldehyde varies, depending on the species and organ being studied (Hedden, 1983; Graebe, 1987). The conversion of GA_{12} -aldehyde → $GA_{12} \rightarrow GA_{53}$ is also microsomal and requires NADPH, which is consistent with a cytochrome P450–dependent enzyme (Kamiya and Graebe, 1982; Hedden, 1983; Graebe, 1987).

Data base comparisons predict that the D3 protein is a member of the cytochrome P450 superfamily. This is consistent with the predicted position of D3 in the pathway ($GA_{12} \rightarrow GA_{53}$); however, the sequence similarity to cytochrome P450 enzymes is equally consistent with any step between *ent*-kaurene and GA_{53}. The predicted D3 protein has the characteristic Fe binding cytosine domain observed in cytochrome P450 proteins (Nebert and Gonzalez, 1987; Porter and Coon, 1991). In addition, the C-terminal 175 amino acids of the predicted D3 protein has ~20% sequence identity with at least 20 known cytochrome P450 proteins.

It is important to test the enzymatic function of the in vitro–expressed D3 protein. If D3 proves to control the conversion of $GA_{12} \rightarrow GA_{53}$, this would establish directly that an early 13-hydroxylation pathway is necessary for shoot elongation in maize, given the dwarf phenotype of d3 plants.

Maize *D3* mRNA expression was observed in multiple tissues. The fact that *d3* plants express the dwarf phenotype at the seedling stage indicates that *D3* expression is required at this stage. This is consistent with finding *D3* mRNA in developing leaves as well as in the roots of 7-day-old maize plants. Note that the *D3* transcript is expressed in roots, because grafting studies using pea GA biosynthetic mutants as scions with wild-type rootstocks have suggested that rootstocks can transmit a putative GA intermediate to the scion (Reid et al., 1983). Furthermore, we found *D3* mRNA in developing leaves and vegetative meristems of 21-day-old plants and suspension culture cells.

One cDNA clone isolated from the vegetative meristem library had an identical 3′ end but showed an altered restriction map relative to the other *D3* clones isolated (R.G. Winkler, unpublished data). Sequence analysis has shown that it is a differentially spliced form of *D3*. The expression of this alternatively spliced *D3* mRNA was not detected in developing leaves or roots. Although there are few reports of alternative splicing in plants, it is a well-known regulatory mechanism in animals.

The isolation of other recently identified genes that control steps in the GA biosynthesis pathway (Sun et al., 1992; Lange et al., 1994; Bensen et al., 1995; Chiang et al., 1995) in combination with maize *D3* will now facilitate a molecular approach to the study of GA biosynthesis. It will be necessary to determine the developmental times and tissues for GA biosynthesis and the ways GA biosynthetic enzymes are regulated to formulate insightful models of how plants use this phytohormone to regulate their growth and development. A number of quantitative trait loci for maize height have been mapped near genes involved in GA biosynthesis and reception. In particular, allelic variation at the *d3* locus has been proposed as the basis of a quantitative trait locus that has been defined for a naturally occurring height variant in maize (Touzet et al., 1995).

METHODS

Plant Material

Seed of maize (*Zea mays*) *dwarf3* (*d3*) alleles were obtained from the Maize Genetics Cooperation Stock Center (Urbana, IL). A stock of *d3-4* that had been backcrossed into A188 seven times was obtained from R. Phillips (University of Minnesota, St. Paul, MN). *d3-660B* was obtained from M. G. Neuffer (University of Missouri, Columbia, MO). Seeds of the maize CM × T recombinant inbred lines were obtained from B. Burr (Brookhaven National Laboratory, Brookhaven, NY). Plants for DNA and RNA preparation were grown in the greenhouse. For mRNA preparation, pools of five tissue sources were extracted: leaves and roots from 7-day-old plants, and leaves and vegetative meristems from 21-day-old plants and NO_3-induced cell cultures (Padgett and Leonard, 1994). All crosses with homozygous dwarf plants were performed in the greenhouse with plants receiving weekly gibberellin A_3 (GA_3) treatments.

DNA Methods

Clones for the *Mutator* elements *Mu1*, *Mu3*, *Mu4*, *Mu5*, and *Mu6* (Chandler and Hardeman, 1991) were obtained from V. Chandler (University of Oregon, Eugene, OR). A PstI-PvuII *Mu8* clone was obtained from S. Hake and R. Walko (U.S. Department of Agriculture, Albany, CA). A *Mu7* clone was obtained from P. Schnable (Iowa State University, Ames, IA). A *MuDR* clone was obtained from D. Lisch and M. Freeling (University of California, Berkeley, CA). Maize restriction fragment length polymorphism (RFLP) clones were obtained from E. Coe (University of Missouri) and B. Burr (Brookhaven National Laboratory). Maize genomic DNA was isolated by the CTAB protocol (Helentjaris et al., 1986) or the protocol of Chen and Dellaporta (1994). Standard techniques were used for restriction enzyme digestion and DNA gel blot transfer (Helentjaris et al., 1986). SeaKem Gold agarose (0.6%) (FMC, Rockland, ME) was used to separate high molecular mass DNA fragments; 3% Metaphor agarose (FMC) was used to separate polymerase chain reaction (PCR) products and other low molecular mass DNA fragments. All DNA gel blots were accomplished by a nonradioactive procedure using probes labeled with 5% digoxigenin-dUTP (Boehringer Mannheim). Most clone inserts were labeled by PCR

amplification of intact plasmids (1 to 10 ng) using primers flanking the cloning site, but longer clones (>1.5 kb) were prepared by oligonucleotide labeling gel-purified inserts using the Genius kit (Boehringer Mannheim). Hybridizations were performed at 65°C in Na_2HPO_4 buffer (0.25 M, pH 7.4), 7% SDS, 1% gelatin, 1 mM EDTA. After a 30-min wash at 65°C in 0.15 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS, blots were processed using the procedures outlined in Boehringer Mannheim catalog No. 101023 V2.0 with minor modifications. AMPPD and CSPD (Tropix, Bedford, MA) were used as alkaline phosphatase substrates.

The following oligonucleotides were used as primers for PCR analysis: G1, 5′-GTCAACATCTCCTTCGTCTCCTTCC-3′; G2, 5′-GAAGTGGTGGAGGAAGACGGAAATC-3′; G3, 5′-TTTCCGTCTTCCTCCACCACTTCC-3′; G4, 5′-GTTTTATTTNGGCACAGACAGGGG-3′; G5, 5′-ACTTTACTATTGGGCTTCCG-3′; G6, 5′-GAGACGAAGGAGATGTTGAC-3′; A1, 5′-CACTGGAATGGTCAAGGCCGGTTTC-3′; A2, 5′-AACCGTGTGGCTCACACCATCACCT-3′.

Genomic Cloning

DNA used in molecular cloning was prepared by the protocol of Chen and Dellaporta (1994). DNA from homozygous *d3-2*::*Mu8* plants was digested with EcoRI and size fractionated on low-melting-temperature agarose gels. Size-selected DNA (10 to 20 kb) was isolated after treatment with β-agarase (FMC) and subsequent EtOH precipitation. The DNA was cloned into λEMBL4 arms (Stratagene) using the *recD*⁻ cell line ER1647 (New England Biolabs, Beverly, MA). Approximately 500,000 primary plaques were screened using the *Mu8* probe, and 10 positive plaques were purified. Two independently isolated positive plaques that had 14-kb inserts and gave identical restriction digest products when digested with EcoRI, BamHI, SalI, and XbaI were recovered. One of these λ clones was subcloned into pBluescript SK− (Stratagene). Genomic restriction mapping of the mutant allele *d3-4* suggested that it had a novel EcoRI site in what was predicted to be an exon of the *D3* gene. To characterize this mutation, the *d3-4* allele was cloned from homozygous *d3-4* plants by techniques similar to that used for *d3-2*::*Mu8*, except that a 5-kb EcoRI fragment was cloned into λZAPII (Stratagene) EcoRI arms using SURE cells (Stratagene) as host. Approximately 500,000 primary plaques were screened with probe 5, and two positive clones were isolated. All subclones used as probes are shown in Figure 2. The two positive 5-kb EcoRI clones were found to be identical by sequence analysis of the 5′ and 3′ ends. The 3′ end of the *d3-4* insertion was cloned by PCR amplification of genomic DNA from homozygous *d3-4* plants with G6, a *D3*-specific primer, and G5, a primer designed from the *Sleepy* insertion near the novel EcoRI site. The resulting ~550-bp band was isolated, reamplified by PCR, and cloned into the plasmid pCRII (Invitrogen, San Diego, CA).

cDNA Cloning

Two amplified λZAP cDNA libraries were screened with probe 26. Both were EcoRI (5′) to XhoI (3′) directionally cloned. A cDNA library prepared from 2-week-old light-grown seedlings (10^6 plaques) (gift of A. Barkan, University of Oregon) yielded two positive plaques with cDNA inserts of 0.8 and 0.9 kb. A cDNA library prepared from vegetative meristems (10^6 plaques) from 4-week-old plants (gift of B. Veits and S. Hake, U.S. Department of Agriculture, Albany, CA) yielded two positive *D3* clones with insert lengths of 1.4 and 1.7 kb.

Sequence Analysis

Overlapping subclones of the genomic and the two longest cDNA clones were prepared using standard techniques. Both strands were sequenced from the following: (1) a 1.4-kb cDNA and the 5′ end of a 1.7-kb cDNA; (2) the novel insertion in *d3-4*; (3) the DNA flanking the *d3-2*::*Mu8* and *d3-4* insertions; (4) the region of *d3-2*::*Mu8* corresponding to the insertion site of *d3-4*; and (5) the region of the *d3-2*::*Mu8* clone corresponding to the 5′ end of the longest cDNA. Plasmid subclones were purified using Wizard minipreparations (Promega). Sequence analysis was performed at the University of Arizona, Tucson, and Iowa State University, Ames, sequencing facilities using Applied Biosystems (Foster City, CA) sequencers.

A data base search and sequence analysis were performed using the Genetics Computer Group (Madison, WI) program (version 8) accessed through the BioScience Computer facility at the University of Arizona. Related sequences were identified by BLAST data base searches, performed at the National Center for Biotechnology Information at the National Library of Medicine (Bethesda, MD) using the BLAST network service (Genbank, release 88). Alignment was performed using the Genetics Computer Group program PILEUP, and the figure was generated with the PRETTYBOX program.

RNA Analysis

Reverse transcription–PCR (RT-PCR; Byrne et al., 1988) was used to evaluate levels of expression of *D3* mRNA. Actin was used as a positive control with the primers A1 and A2. The 3′ end of the actin A1 primer spans the first intron, and the 3′ end of the A2 primer spans the second intron. Genomic DNA is not amplified with these oligonucleotides because the 3′ ends of the primers are not complementary to the genomic DNA sequence. G1/G2 and G3/G4 were the *D3* primer pairs used. The *D3* primer pairs were designed for PCR amplification of a region that contains an intron and therefore would differentiate between genomic DNA (G1/G2, 334-bp PCR product; G3/G4, 374-bp product) and the cDNA (G1/G2, 260-bp product; G3/G4, 258-bp product).

Total RNA was purified using the guanidine thiocyanate method (Chomczynski and Sacchi, 1987). Contaminating genomic DNA was removed by treatment with RNase-free DNase I (GeneHunter, Brookline, MA). First-strand cDNA was synthesized using 5 μg of total RNA at 50°C for 2 hr using oligo(dT) (n = 15) and SuperScript II (Bethesda Research Laboratories) with the buffer conditions suggested by the manufacturer (total volume of 50 μL). PCR was performed with 2 μL of the first-strand cDNA reaction. The reaction was initially denatured at 95°C for 2 min and in the 30 subsequent cycles at 94°C for 30 sec; annealing cycles were 30 sec long at 65°C, and elongation cycles were 90 sec long at 72°C.

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